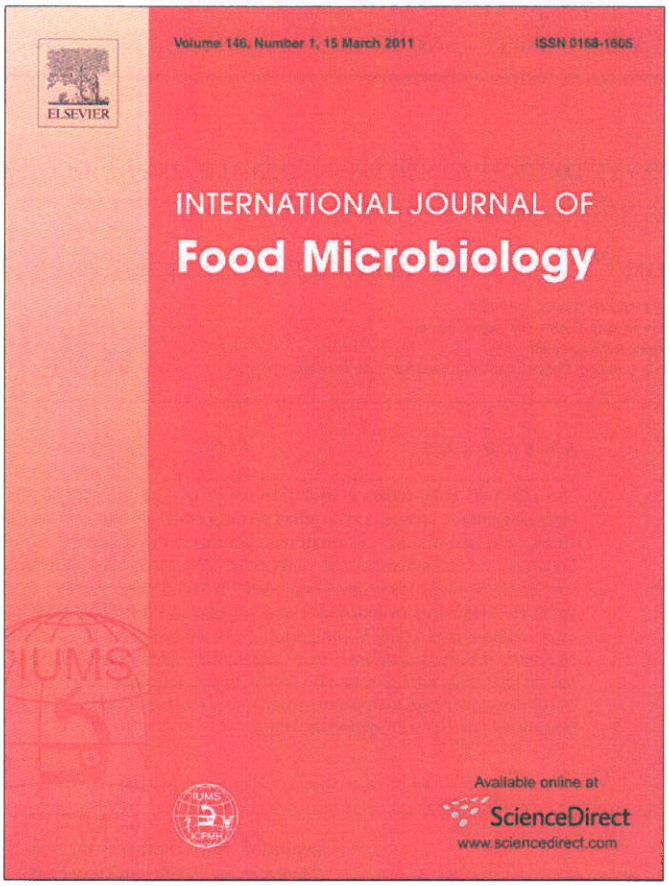

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Glycine betaine improves oxidative stress tolerance and biocontrol efficacy of the antagonistic yeast *Cystofilobasidium infirmominiatum*

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ABSTRACT

The effect of H_2O_2-induced oxidative stress on the viability of the yeast antagonist, *Cystofilobasidium infirmominiatum*, as well as the effect of exogenous glycine betaine (GB) on yeast viability under oxidative stress, was determined. GB treatment improved the tolerance of *C. infirmominiatum* to oxidative stress. Compared to untreated control yeast cells, GB-treated cells showed less accumulation of reactive oxygen species (ROS) and a lower level of protein oxidation in response to oxidative stress. Additionally, GB-treated yeast exhibited greater biocontrol activity against *Penicillium expansum* and a faster growth in wounds of apple fruits stored at 25 °C compared to the performance of untreated yeast. The activities of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) of *C. infirmominiatum* were elevated by GB treatment. Results indicate that the elicitation of antioxidant response by GB may contribute to improvements in oxidative stress tolerance, population growth in apple wounds, and biocontrol activity of *C. infirmominiatum*.

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1. Introduction

In recent years, the use of antagonistic microbial agents to control postharvest pathogens has shown great potential (Droby et al., 2009; Spadaro and Gullino, 2004). Many yeast species have been reported to be effective antagonists against postharvest diseases of fruits and vegetables (Sharma et al., 2009; Wisniewski et al., 2007). Among yeast antagonists, *Cystofilobasidium infirmominiatum* (Fell, I.L. Hunter & Tallman) Hamam., Sugiy. & Komag (anamorph: *Cryptococcus infirmominiatus* (Okunuki) Phaff et Fell) has demonstrated biocontrol efficacy against postharvest diseases of several fruits, including pear, apple and sweet cherry (Chand-Goyal and Spotts, 1997; Spotts et al., 2002; Spotts et al., 2009; Vero et al., in press).

During the application of biocontrol agents, it is inevitable for them to face a wide array of environmental conditions that may affect viability and efficacy. Oxidative stress, one of the most common stresses, plays a crucial role in biocontrol systems (Castoria et al., 2003; Macarisin et al., 2010). The ability to survive and proliferate in wounded host tissues is pivotal for postharvest biocontrol agents (Droby et al., 2009; Janisiewicz and Korsten, 2002), and wounding of fruit tissue is associated with the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anions that can effect host response (Macarisin et al., 2010; Torres et al., 2003), pathogen virulence (Lu and Higgins, 1999; Macarisin et al., 2007) and yeast efficacy. (Castoria et al., 2003; Tolaini et al., 2010). Thus, enhancing tolerance to oxidative stress may represent a useful strategy for improving the effectiveness of biocontrol yeasts.

Glycine betaine (GB, *N, N, N*-trimethyl glycine) is one of the most common compatible solutes that contribute to osmotic adjustment for bacteria, fungi, algae, plants, and animals (de Zwart et al., 2003; Rhodes and Hanson, 1993). GB stabilizes the structure and activity of enzymes, as well as the integrity of membranes against salt/osmotic stress (Ashraf and Foolad, 2007; Boncompagni et al., 1999; Wang et al., 2007). In addition to its role as osmoprotectant, GB has been found to provide a protective function by inducing antioxidant response under stress conditions. Exogenous application of GB enhanced antioxidant enzyme activity and conferred tolerance of cultured tobacco cells to sodium chloride- and cadmium-induced stresses (Hoque et al., 2008; Islam et al., 2009). GB has also been reported to induce antioxidant defense responses in other plant species, such as wheat (Raza et al., 2007), rice (Farooq et al., 2008), tea (Kumar and Yadav, 2009) and mung bean (Hossain and Fujita, 2010). Although GB has been reported to be an effective osmoprotetant for yeasts like *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Thomas et al., 1994) and *Pichia pastoris* (Guillierm.)

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Phaff (Wang et al., 2007), and for bacteria such as *Escherichia coli* (Migula) Castellani and Chalmers (Cayley and Record, 2003) and *Gluconacetobacter diazotrophicus* Yamada, Hoshino and Ishikawa (Boniolo et al., 2009), there is little information about the effect of GB on antioxidant systems in microorganisms.

The objective of the present study was to determine the effect of GB on oxidative stress tolerance and biocontrol efficacy of the yeast antagonist, *C. infirmominiatum*. More specifically, we investigated (i) the viability of *C. infirmominiatum* in response to exposure to a range of oxidative stress conditions stimulated by varying concentrations of hydrogen peroxide, (ii) the effect of exogenous GB on oxidative stress tolerance, ROS accumulation and protein oxidation, (iii) the effect of GB on population dynamics and biocontrol efficacy of *C. infirmominiatum* against *Penicillium expansum* Link on apple fruits, and (iv) the effect of GB on induction of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) in *C. infirmominiatum*.

2. Materials and methods

2.1. Yeast

C. infirmominiatum (PL1) was obtained from the culture collection maintained by Dr. Silvana Vero at the University of the Republic, Uruguay. Twenty milliliters of yeast peptone dextrose (YPD consisting of 10 g of yeast extract, 20 g of peptone and 20 g of dextrose in 1000 ml water) was prepared in 50-ml conical flasks and inoculated with *C. infirmominiatum* at an initial concentration of 10^5 cells/ml determined by an automated cell counter, Cellometer Vision (Nexcelom Bioscience, Lawrence, MA, USA). Yeast cultures were incubated at 25 °C on a rotary shaker at 200 rpm. All media components were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Pathogen

P. expansum was isolated from infected apple fruit and maintained on potato dextrose agar (PDA) (Difco, Sparks, MD, USA) at 4 °C. The pathogen was inoculated into apple fruit wounds and re-isolated onto PDA prior to use. Spore suspensions were obtained from 2-week-old cultures on PDA at 25 °C. The number of spores was calculated using a Cellometer Vision (Nexcelom Bioscience, Lawrence, MA, USA), and the spore concentration was adjusted to 1×10^4 spores/ml with sterile distilled water.

2.3. Fruits

'Golden Delicious' apples (*Malus × domestica* Borkh.) were harvested at commercial maturity. Fruits without wounds or rot were selected based on uniformity of size, disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water and dried in air according to the method of Yao et al. (2004). These fruits were used in a subsequent biocontrol assay.

2.4. Evaluation of survival of C. infirmominiatum under oxidative stress

Overnight yeast cultures were pelleted at 8000 *g* for 3 min and washed three times with sterile distilled water in order to remove residual medium, according to Li and Tian (2006). A 10 ml yeast cell suspension at 5×10^7 cells/ml in water was placed in 50-ml conical flask. The yeast cells were then exposed to a final concentration of 5, 10 or 20 mM H_2O_2 at 25 °C on a shaker at 200 rpm for 20, 40 or 60 min. At the described time points, yeast cells were collected by centrifugation and washed once with water, then 50 µl of serial 10-fold dilutions (from 5×10^6 to 5×10^3 cells/ml) of the samples were spread on YPD agar plates. The plates were incubated at 25 °C for 3 days and the number of colony-forming units per milliliter (CFU/ml) was calculated. Survival rates were expressed as a percentage of the number of colonies after the oxidative treatment relative to the number of colonies before the treatment. There were three replicates in each treatment, and each experiment was repeated three times.

2.5. Effect of GB on oxidative stress tolerance of C. infirmominiatum

The effect of GB on survival of *C. infirmominiatum* after H_2O_2 treatment was determined according to the method of Deveau et al. (2010), with slight modification. Washed cells from overnight culture were resuspended in the same volume of fresh YPD, supplemented with GB at the final concentration of 1 mM (the concentration was selected based on previous studies (Boncompagni et al., 1999; Bouvier et al., 2000) and our preliminary unreported data) and incubated at 25 °C for 2 h at 200 rpm. Yeast cells without GB treatment served as a control. Cells were then harvested by centrifugation at 8000 *g* for 3 min and washed three times with sterile distilled water in order to remove any residual GB and medium. Then, GB-treated yeast and control samples at 5×10^7 cells/ml were exposed to 10 mM H_2O_2 at 25 °C at 200 rpm for 20, 40 or 60 min, and survival rates at each time point were evaluated with the same method and replications described above.

2.6. Imaging of intracellular reactive oxygen species

The oxidant-sensitive probe, 2 ,7 -dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Eugene, OR, USA), was used to assess the intracellular ROS production in *C. infirmominiatum* according to Kim et al. (2010), with a slight modification. Yeast cells (control or GB-treated) were collected from samples exposed to 10 mM H_2O_2 at 0, 20, 40 and 60 min. Cells were washed with phosphate buffered saline (PBS) buffer (pH 7.0) and resuspended in the same buffer containing 25 µM H_2DCFDA (dissolved in dimethyl sulfoxide). The suspension was incubated in the dark at 30 °C for 1 h. After washing twice with PBS buffer, spores were examined under a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) equipped with a UV-light source using a 485-nm excitation and 530-nm emission filter combination. Five fields of view from each slide (at least 200 cells) were randomly chosen, the number of cells producing visible levels of ROS in response to oxidative stress was counted for both control and GB-treated samples. The ROS level was calculated as a percentage (number of fluorescing cells divided by number of cells present in bright field image X 100). There were three replicates in each treatment, and the experiment was repeated three times.

2.7. Assay of protein oxidation

Protein oxidation was measured by determining carbonyl content (Fagan et al., 1999; Hoque et al., 2008). Yeast cells (control or GB-treated) were collected from samples exposed to 10 mM H_2O_2 for 0, 20, 40 and 60 min. Yeast cells were disrupted in liquid nitrogen by grinding in a mortar with a pestle, and proteins were extracted from the samples using 50 mM KH_2PO4 buffer (pH 7.5) containing 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA and 1 mM PMSF. Aliquots of extract were reacted with 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 M HCl or 2.5 M HCl without DNPH (blank control) in the dark at room temperature with vortex every 15 min for 1 h. Proteins were precipitated with 20% trichloroacetic acid (TCA, w/v) and kept on ice for 10 min. After centrifuging at 3,000 g for 20 min, protein pellets were washed with ethanol-ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine hydrochloride with 20 mM KH_2PO_4 (pH 2.3). The absorbance was recorded at 380 nm after centrifugation at 9,500 g for 10 min. The carbonyl content was calculated using the molar absorption coefficient of 22,000/M/cm, and expressed as nmol/mg protein. Protein content was measured using the Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a

standard. There were three replicates in each treatment, and the experiment was repeated three times.

2.8. Determination of the enzyme activities of CAT, SOD and GPX

Extracts for enzyme assays of CAT, SOD and GPX were prepared according to Liu et al. (2005). Washed cells from overnight culture were resuspended in the same volume of fresh YPD, supplemented with GB at the final concentration of 1 mM and incubated at 25 °C for 2 h at 200 rpm. The yeast cells without GB treatment served as control. The cells were then harvested by centrifugation at 8000 g for 3 min and washed three times with sterile distilled water in order to remove residual GB and medium. Yeast cells were then exposed to 10 mM H_2O_2 at 25 °C at 200 rpm for 1 h. The yeast samples at each time point (0, 1, 2 h during GB treatment, and another 1 h after exposure to H_2O_2) were collected by centrifugation at 8000 g for 3 min and washed three times with sterile distilled water. Cells were disintegrated in liquid nitrogen and suspended in chilled potassium phosphate buffer (0.1 M, pH 7.4). The cell homogenate was centrifuged at 10,000 g for 20 min at 4 °C, and the supernatant was used for enzyme assays. The activities of SOD, CAT and GPX were assayed and expressed as U/mg protein, according to the previous study (Reverberi et al., 2005). One unit of CAT activity was defined as the decomposition of 1 μmol H_2O_2 per minute. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition in the nitro blue tetrazolium (NBT) reduction. One unit of GPX activity was defined as the oxidation of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) per minute. Protein content was measured as described by Bradford (1976), using bovine serum albumin (BSA) as a standard. There were three replicates in each treatment, and the experiment was repeated three times.

2.9. Biocontrol assay of C. infirmominiatum against P. expansum on apple fruits

Biocontrol activity of *C. infirmominiatum* was tested according to a previous study (Liu et al., 2009). Three wounds (4 mm deep × 3 mm wide) were made on the equator of each fruit with a sterile nail. A 10 μl suspension of non-GB-treated (NGB) or GB-treated (GB) cells of *C. infirmominiatum* (5×10^7 cells/ml) was applied to each wound. Sterile distilled water served as a control. After fruits were air-dried for 2 h, 10 μl of *P. expansum* suspension (1×10^4 spores/ml, 2-week-old fresh culture) was inoculated into each wound. Treated fruits were placed in a covered plastic food tray, and each tray was enclosed with a polyethylene bag and stored at 25 °C. Disease incidence and lesion diameter of apple fruits caused by *P. expansum* were determined after 4 days. Each treatment contained three replicates where a replicate consisted of ten fruits with three wounds per apple. The average percent infection was calculated for each replicate (number of wounds with infection/30 × 100). The experiment was repeated three times and the overall percent infection was calculated as the average of the percent infection of nine replicates. Incidence represented the percentage of fruit displaying rot while lesion diameter was measured only on those wounds that were infected.

2.10. Population dynamics of C. infirmominiatum in wounds of apple fruits

Three wounds (4 mm deep × 3 mm wide) were made on the equator of each apple fruit with a sterile nail. A 10 μl suspension of NGB or GB cells of *C. infirmominiatum* (5×10^7 cells/ml) was applied to each wound. Fruit samples were collected at different time points after treatment and yeast populations were measured as described by Cao et al. (2010). Briefly, yeasts were recovered by removing ten samples of wounded tissues with a cork borer (1 cm diameter × 1 cm deep). Samples were then ground with a mortar and pestle in 10 ml sterile distilled water. Then, 50 μl of serial 10-fold dilutions were spread on YPD agar plates. Samples taken at 1 h after treatment served as time 0. Fruits stored at 25 °C were assessed each day for 4 days. Colonies were counted after incubation at 25 °C for 3 days and expressed as the Log_{10} CFU per wound. There were three replicates in each treatment, and the experiment was repeated three times.

2.11. Data analysis

All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Two-way ANOVA analysis was performed on data where treatment and time were variables, and mean separation was determined for non-GB-treated control (NGB) and GB at each time point using a Student's *t*-test. Data with a single variable (treatment) were analyzed by one-way ANOVA, and mean separations were performed by Duncan's multiple range tests. Differences at $P<0.05$ were considered significant. Data presented in this paper were pooled across three independent repeated experiments.

3. Results

3.1. Survival of C. infirmominiatum under H_2O_2-induced oxidative stress

As expected, the viability of *C. infirmominiatum* cells decreased with increasing H_2O_2 concentration and exposure time within each concentration (Table 1). Under the oxidative stress of 5 mM H_2O_2 for 20 min, yeast cells exhibited 93.2% viability. At 20 mM H_2O_2, *C. infirmominiatum* cells had a viability of 23.3% at 20 min and only 5.8% at 60 min. Compared to concentrations of 5 and 20 mM H_2O_2, yeast cells exhibited intermediate levels of survival in 10 mM H_2O_2 at all treatment time points. Based on the result of the viability assay, 10 mM H_2O_2 was chosen to be the appropriate stress condition for assessing the effect of GB treatment on improving oxidative stress tolerance.

3.2. Effect of GB on oxidative stress tolerance of C. infirmominiatum

Both GB treatment and duration of exposure to the oxidative stress had a significant ($P<0.05$) effect on *C. infirmominiatum* viability. As indicated in Fig. 1, the viability of yeast cells exposed to 10 mM H_2O_2 decreased over the exposure time range (20–60 min). However, GB-treated cells had a significantly higher viability compared to control cells at all three time points. For example, after 40 min at 10 mM H_2O_2, survival of control yeast cells was 58.6%, while that of GB-treated cells was 75.4%.

3.3. ROS accumulation and protein oxidation of C. infirmominiatum under oxidative stress

As illustrated in Fig. 2, at time 0, prior to H_2O_2 treatment, the percentages of both GB and control cells exhibiting a visible ROS level, as determined by use of the fluorescent dye H_2DCFDA, were less than 5%. However, the percentage increased with treatment time exposure

Table 1
Percent viability of *C. infirmominiatum* under oxidative stress.

H_2O_2 (mM)	Treatment time (min)		
	20	40	60
5	93.2 ± 3.6a	84.7 ± 3.1a	76.5 ± 2.8a
10	72.1 ± 2.5b	51.5 ± 4.3b	31.7 ± 3.0b
20	23.3 ± 2.9c	14.4 ± 2.3c	5.8 ± 2.0c

Values are the means of pooled data ± standard deviations (n = 9). The values followed by different letters at each treatment time point are significantly different according to Duncan's multiple range test ($P<0.05$).

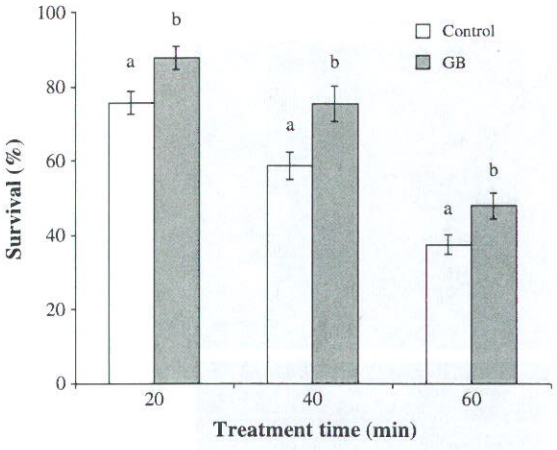

Fig. 1. Percent survival of control (non-GB-treated) and GB-treated yeast cells of *C. infirmominiatum* exposed to oxidative stress (10 mM H_2O_2) for varying periods of time. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9). Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

to 10 mM H_2O_2. Both GB treatment and duration of exposure to the oxidative stress had a significant ($P<0.05$) effect on intracellular ROS accumulation in *C. infirmominiatum*. GB-treated cells exhibited a significantly lower percentage of cells exhibiting a visible ROS level compared to control cells at all three time points (after time 0) under the oxidative stress. After 60 min at 10 mM H_2O_2, the percentage of GB-treated cells stained with H_2DCFDA was 53.4% while that of control cells was 67.1%. While we could not determine if fluorescent cells were living or dead, the percentage of non-fluorescent cells was very close to the percentage obtained in the viability assay conducted in other portions of this study (Fig. 1). Therefore, it appeared that the percentage of fluorescent cells was negatively correlated with cell viability.

To investigate the protective effect of GB against H_2O_2-induced protein oxidation, carbonyl content in *C. infirmominiatum* cells was measured (Fig. 3). The change in levels of protein oxidation was similar to that of ROS accumulation. At time 0, prior to H_2O_2 treatment, carbonyl content in both control and GB-treated cells was quite low (less than 0.3 nmol/mg protein). Both GB treatment and duration of exposure to the oxidative stress had a significant ($P<0.05$) effect on carbonyl content in *C. infirmominiatum*. Oxidative stress (10 mM H_2O_2) resulted in a marked increase in carbonyl content in *C. infirmominiatum* cells over the exposure time. However, GB-treated cells had significantly lower carbonyl content than control cells at all three time points after time 0. After 60 min at 10 mM H_2O_2, carbonyl content of control yeast cells was 2.6 nmol/mg protein, while that of GB-treated cells was 2.0 nmol/mg protein.

3.4. Activities of antioxidant enzymes

GB treatment enhanced the enzyme activities of CAT, SOD and GPX in *C. infirmominiatum* cells, and this inductive effect could be observed for at least 1 h after exposure to 10 mM H_2O_2 (Fig. 4). CAT activity exhibited a gradual increase during the 2-h GB treatment, and the activity level was almost 1.5-fold greater than CAT activity in control cells at 2 h. When exposed to H_2O_2 for 1 h, CAT activity of both GB-treated and control cells increased markedly, however, the activity was also higher in GB-treated cells than in control cells (Fig. 4A). GB treatment had little inductive effect on SOD activity at 1 h, but the activity in GB-treated cells was significantly higher than that in control at 2 h, and also at 1 h after exposure to H_2O_2 (Fig. 4B). GPX activity in control cells was relatively stable but increased dramatically after exposure to H_2O_2. GB-treated cells showed higher activity than control cells at each time point after time 0 (Fig. 4C).

3.5. Biocontrol efficacy and population dynamics of C. infirmominiatum

As shown in Fig. 5, *C. infirmominiatum* effectively controlled postharvest disease caused by *P. expansum* on apple fruits stored at 25 °C. However, GB-treated yeast exhibited a greater level of efficacy compared to NGB-treated yeast. Disease incidence in apple fruits treated with NGB-treated vs. GB-treated cells was 41.1% and 26.7%, respectively, while disease incidence in the control fruits (inoculated with water followed by the pathogen) reached 100%. Additionally, lesion diameter was significantly smaller on apples treated with GB-treated cells ($P<0.05$).

C. infirmominiatum multiplied quickly in apple fruit wounds (Fig. 6), and after 1 day, the number of the yeast increased more than ten-fold. The population of GB-treated cells was significantly ($P<0.05$) higher than the population of NGB-treated cells at 1 and 2 days. However, the difference between GB-treated and NGB-treated cells was no longer significant by 3 days when cells reached the stationary phase. The effect of GB treatment on yeast growth in apple wounds over all time points was significant ($P<0.05$).

4. Discussion

Improving the stress tolerance of biocontrol agents is a useful strategy for maintaining or increasing the performance of postharvest biocontrol agents under practical conditions (Teixidó et al., 2006; Wang et al., 2010). In the present study, GB has been shown to be effective in improving the tolerance of *C. infirmominiatum* to oxidative stress, an important factor influencing the survival and efficacy of biocontrol yeasts. For example, Castoria et al. (2003) reported that the viability of *Rhodotorula glutinis* (Fresen.) Harrison decreased with increasing duration of oxidative stress, and Patiño-Vera et al. (2005) postulated that during storage of *Rhodotorula minuta* (Saito) Harrison in liquid formulation, oxidative stress affected cell viability. Both yeast species have been used in biocontrol studies. In the present study, we observed that oxidative stress also had a significant effect on the viability of *C. infirmominiatum*. The effect on viability increased with time at any specific level of oxidative stress. Yeast viability was quite low after exposure to 20 mM H_2O_2 (Table 1).

It has been well known that glycine betaine (GB) is a common osmoprotectant for organisms. Additionally, in recent years, glycine betaine has been reported to have a significant antioxidant effect and improve stress tolerance in many plants (Farooq et al., 2008; Hossain and Fujita, 2010; Kumar and Yadav, 2009; Raza et al., 2007), as well as animal cells (Alfieri et al., 2002; Monobe et al., 2006). In our study, it was found that GB-treatment increased the viability of *C. infirmominiatum* under oxidative stress (10 mM H_2O_2) (Fig. 1). In previous studies, exogenous GB was found to have a protective effect on the viability of *S. cerevisiae* under ethanolic fermentation (Thomas et al., 1994), and the expression of glycine betaine synthesis genes from *Suaeda salsa* L. in *P. pastoris* improved yeast resistance to salt, methanol and high temperature stresses (Wang et al., 2007). In addition, Cañamás et al. (2007) found that the biocontrol agent *Pantoea agglomerans* could synthesize significant amounts of GB and ectoine in response to an imposed solute stress. Furthermore, Teixidó et al. (2005) reported that modifying the water activity of the growth medium induced the accumulation of intracellular GB and ectoine in *P. agglomerans*, resulting in the improvement of osmotic and thermal tolerance. These studies indicate that both exogenous and intracellular GB can play a positive role in stress tolerance.

When yeasts are exposed to severe stresses including oxidative stress, large amounts of intracellular ROS are generated. Accumulation of ROS can cause oxidative damage to cell components including proteins, lipids and nucleic acids, resulting in a decrease in cell

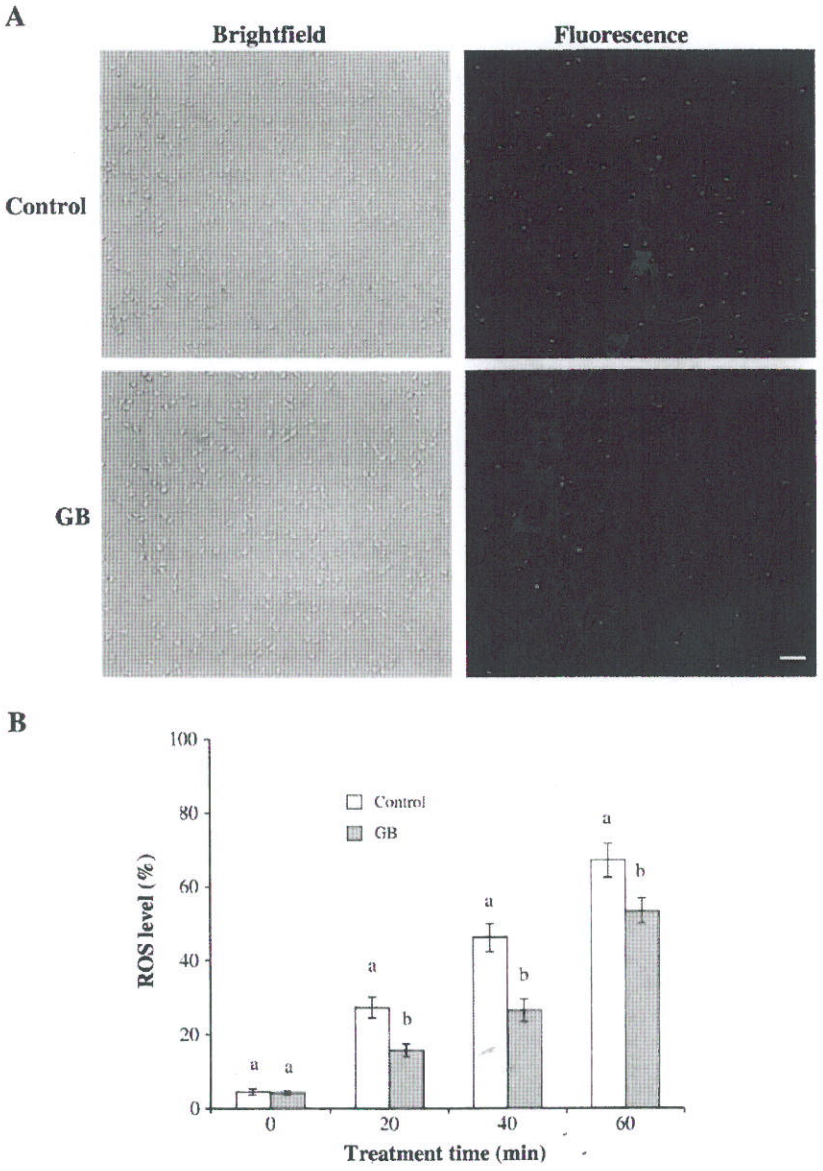

Fig. 2. ROS accumulation in control and GB-treated cells of *C. infirmominiatum* exposed to oxidative stress (10 mM H_2O_2) for varying periods of time. (A) microscopy images of *C. infirmominiatum* cells after 60 min at 10 mM H_2O_2 as observed under bright field and UV-light. ROS was visualized using the fluoroprobe H_2DCFDA, and 485-nm excitation and 530-nm emission filter combination; (B) Percentage of *C. infirmominiatum* cells exhibiting visible ROS accumulation after exposure to 10 mM H_2O_2 for varying periods of time. Scale bar (—) represents 20 μm. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9). Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

viability (Branduardi et al., 2007; Reverter-Branchat et al., 2004). We found that the lower percentage of GB-treated cells exhibiting visible levels of ROS (as determined with the fluoroprobe H_2DCFDA, compared to NGB-treated control cells) (Fig. 2), was associated with the higher viability of GB-treated yeast cells exposed to oxidative stress stimulated by 10 mM H_2O_2 (Fig. 1).

Protein oxidation occurs frequently in cells under oxidative stress and has a deleterious effect on protein structure and function. The level of carbonyl groups of proteins can be used as a marker of oxidative protein damage (Abegg et al., 2010; Stadtman and Levine, 2003). To further verify whether or not such oxidative modification occurred when *C. infirmominiatum* was under H_2O_2-induced oxidative stress, we studied the carbonyl content of proteins. The data in Fig. 3 indicated that lower carbonyl content was detected in GB-treated cells under oxidative stress, compared to control cells. These findings are consistent with results obtained by Hoque et al. (2008), who found that GB treatment obviously reduced protein carbonylation of tobacco cells under salt stress. Taken together, these data indicated that GB treatment reduced oxidative damage and increased the viability of *C. infirmominiatum* cells under H_2O_2-induced oxidative stress. This may be due to the function of glycine betaine recently reported by Einset and Connolly (2009), suggesting that GB blocks ROS signaling by activating antioxidant enzymes thus reducing the level of ROS available to trigger ROS-related cellular responses.

The detoxification of ROS is dependent on antioxidant enzymes such as CAT, SOD and GPX. The activity increase of these enzymes

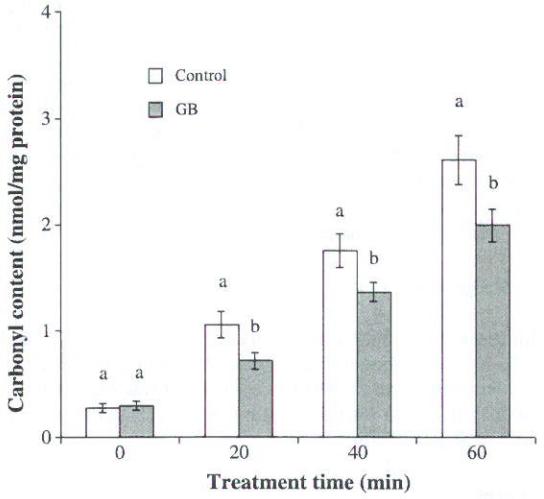

Fig. 3. Protein oxidation in control and GB-treated cells of *C. infirmominiatum* exposed to oxidative stress (10 mM H_2O_2) for varying amounts of time. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9). Columns with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

contributes to the adaptation of yeast cells to oxidative stress and ameliorates oxidative damage (Abegg et al., 2010; Liu et al., 2005). In the present study, we also found that the activities of the three enzymes of *C. infirmominiatum* cells increased markedly when exposed to H_2O_2 (Fig. 4). This result confirmed the previous finding by Pinheiro et al. (2002), who reported the treatment of H_2O_2 for 1 h induced activities of CAT, SOD and glutathione reductase in *Kluyveromyces marxianus* (Hansen) Van der Walt. Importantly, GB treatment alone stimulated the activities of CAT, SOD and GPX in *C. infirmominiatum* cells both before and under H_2O_2-induced oxidative stress (Fig. 4). Similar results have been reported in previous studies. Farooq et al. (2008) demonstrated that exogenous GB improved drought tolerance of rice by elicitation of CAT, SOD and ascorbate peroxidase. Hossain and Fujita (2010) reported that GB enhanced the activities of GPX and other enzymes related to ROS metabolism as well as the methylglyoxal detoxification system in mung bean seedlings. We suggest that the GB induction of antioxidant enzyme activity in *C. infirmominiatum* may be a key factor in lowering ROS levels and protein oxidation, thus improving the viability of yeast cells exposed to oxidative stress.

In addition to improved oxidative stress tolerance *in vitro*, biocontrol efficacy of *C. infirmominiatum* was also enhanced by the GB-treatment (Fig. 5). High population density is an advantage to microbial antagonists in competing for nutrients and space, both of which play a major role in biocontrol efficacy (Droby et al., 1989; Wisniewski et al., 2007). It is notable that GB-treated cells of *C. infirmominiatum* multiplied more quickly than NGB-treated cells in wounds of apple fruits during the first two days after inoculation (Fig. 6). This observation is in agreement with the stimulating effect of GB on growth of microorganisms exposed to abiotic stress (Boniolo et al., 2009; Delamare et al., 2003; Thomas et al., 1994). The period of the first two days after inoculation in apple wounds is important for the germination and infection of *P. expansum* (Li et al., 2008). Thus, the higher population of GB-treated yeast cells in this period may have led to the better biocontrol ability, as compared to NGB-treated cells. Additionally, the fruit wound environment is characterized by a high level of ROS, and oxidative stress resistance could be a key factor in the efficacy of biocontrol yeasts. Castoria et al. (2003) reported that oxidative stress resistance was necessary for biocontrol yeasts to remain viable in wounded fruit. Macarisin et al. (2010) found that

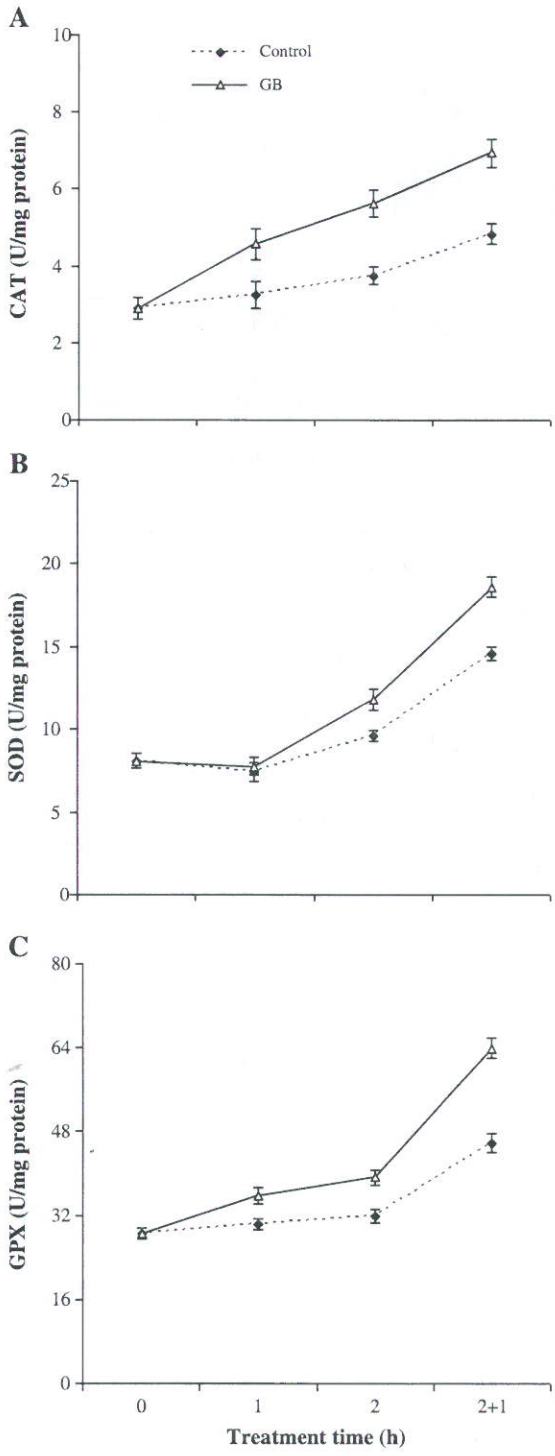

Fig. 4. Change of CAT (A), SOD (B) and GPX (C) activities in control and GB-treated cells of *C. infirmominiatum*. Yeast cells of overnight culture were treated with 1 mM GB for 2 h, then washed and exposed to 10 mM H_2O_2 for 1 h. Cells without GB treatment served as control. The enzyme activities of yeast samples at each time point (0, 1, 2 h during GB treatment, and another 1 h after exposure to H_2O_2) were assayed. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9).

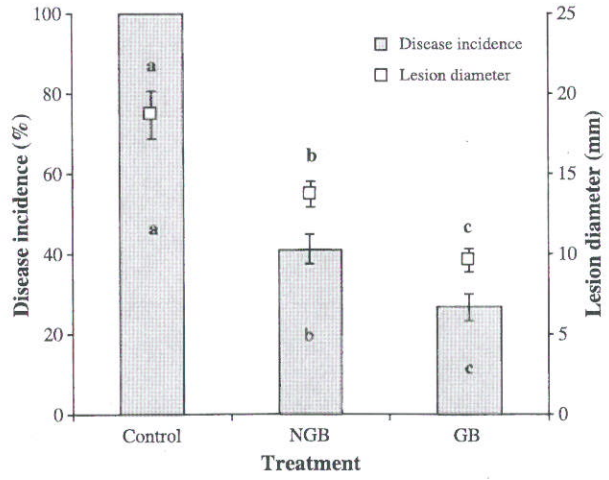

Fig. 5. Biocontrol efficacy of *C. infirmominiatum* against *P. expansum* decay on apple fruits stored at 25 °C. Fruits were wounded and inoculated with 10 μl of sterile water (control), non-GB-treated (NGB) or GB-treated (GB) cell suspension of *C. infirmominiatum* at 5×10^7 cells/ml. After 2 h, wounds were inoculated with 10 μl of *P. expansum* at 1×10^4 spores/ml. Disease incidence and lesion diameter were detected 4 days after inoculation with *P. expansum*. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9). Columns with different letters indicate significant differences according to Duncan's multiple range test ($P<0.05$).

yeasts underwent an oxidative burst when applied to fruit, and induced host cells in wounded fruit tissue to produce elevated levels of ROS as part of a presumed reaction leading to the production of defense substances. In this study, therefore, the GB-induced oxidative stress tolerance may contribute to the improvement of population dynamics and biocontrol efficacy of *C. infirmominiatum* in apple wounds.

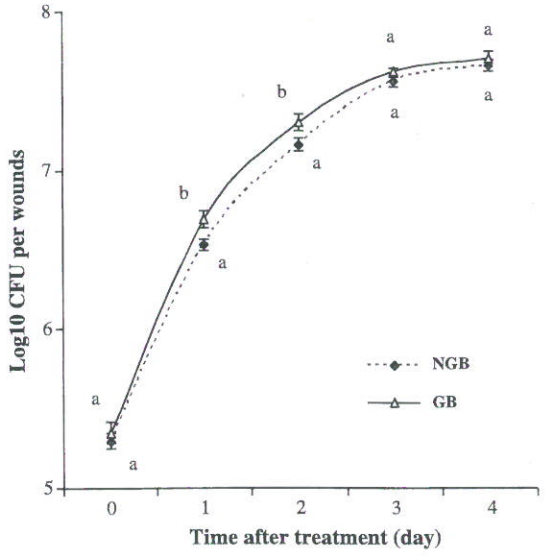

Fig. 6. Population dynamics of *C. infirmominiatum* in wounds of apple fruits stored at 25 °C. Fruits were wounded and inoculated with 10 μl of non-GB-treated (NGB) or GB-treated (GB) cell suspension of *C. infirmominiatum* at 5×10^7 cells/ml. Population levels were assessed each day for 4 days. Yeast colonies were counted after incubation at 25 °C for 3 days and expressed as the Log_{10} CFU per wound. Data presented are the means of pooled data. Error bars indicate standard deviations of the means (n = 9). Symbols with different letters at each time point indicate significant differences according to Student's *t*-test ($P<0.05$).

In conclusion, we found that glycine betaine could improve oxidative stress tolerance of *C. infirmominiatum*, and reduce intracellular ROS accumulation and protein oxidation when yeast cells were exposed to oxidative stress. The improvement in oxidative stress tolerance by GB treatment was associated with an induction of antioxidant enzyme activity. This is the first report of improvement of *in vivo* growth and biocontrol efficacy of an antagonistic yeast by GB. These results may have practical implications for the application of GB for improving the viability and efficacy of biocontrol agents used to manage postharvest diseases of fruits.

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